# THE PRODUCTION OF EXTRACELLULAR PROTEASE USING *BACILLUS SUBTILIS* : EFFECT OF TEMPERATURE AND AGITATION SPEED

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## DECLARATION

I declare that this thesis entitled "The Production of Extracellular Protease using *Bacillus subtilis:*the Effect of Temperature and Agitation Speed " is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : .....

Name : MOHD FADLI BIN ABDULLAH

Date : 20 NOVEMBER 2006

**DEDICATION**

To my beloved mother, father, my siblings
and friends

# ACKNOWLEDGEMENT

In preparing this thesis, I was in contact with many people who have contributed in ensuring the success of this project. First and foremost, I would like to express my appreciation and gratitude to my supervisor, En. Wan Mohd Hafizuddin bin Wan Yussof for guiding me throughout this study. Thank you for broading my knowledge to the world of microbiology/biotechnology. Thank you for the encouragement, guidance, and critics. The same goes to En. Rozaimi bin Abu Samah, my co-supervisor. Both of you have have made this thesis what it is today.

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## ABSTRACT

Production of extracellular protease refers to proteases produced from microorganisms and functions out of the cell in which it was originated. In this term, the microorganism was sought to be normal mesophilic bacteria. The experiment was conducted to evaluate the effects of temperature and agitation speed of the incubator shaker towards the production of extracellular protease. Optimization of the enzyme production condition involving the two parameters was also made using statistical software. Typical methods conducted in previous researches were utilized in the experiments using different temperature and agitation speed. However, in previous researches, experiments were conducted through one-factor-at-a-time method (OFAT) but in this experiment, that method was overrun and designed beforehand using Minitab, statistical software with response surface design. The standard procedures of protease assay were carried out when experimenting. The bacteria were re-cultured to enable its functionality to serve its purpose. The results obtained proved that both parameters had significant effects if stood alone towards the production, however had to interact with each other due to randomization of the values implemented in the experiments. Simple analysis was not an option therefore the results were opted to be analyzed by Minitab and also the condition of protease production was optimized.

# ABSTRAK

Penghasilan atau penjanaan protease ekstra selular merujuk kepada protease yang terhasil daripada mikroorganisma dan berfungsi di luar sel yang menjadi perumahnya. Dalam konteks ini, mikrorganisma yang digunakan merupakan mesofil yang normal. Eksperimen dijalankan untuk mengkaji kesan suhu dan kelajuan agitator incubator shaker terhadap penghasilan protease ekstra selular. Perisian statistik telah digunakan untuk mewujudkan keadaan optimum untuk penghasilan enzim tersebut melibatkan kedua-dua parameter eksperimen. Kaedah yang tipikal dalam kajian terdahulu telah digunakan dalam eksperimen menggunakan suhu dan kelajuan putaran yang berbeza. Walaubagaimanapun, dalam kajian terdahulu, eksperimen telah dijalankan melalui one-factor-at-a-time method (OFAT), menetapkan satu parameter dan memanipulasikan parameter yang lain. Dalam eksperimen ini, kaedah tersebut tidak digunakan lagi malah eksperimen telah direka terlebih dahulu menggunakan Minitab, perisian statistik dengan rekaan Response Surface. Prosedur standard dalam protease assay telah diikuti semasa menjalankan eksperimen. Bakteria telah dikultur semula untuk membolehkannya berfungsi dengan sempurna. Keputusan eksperimen membuktikan bahawa kedua-dua parameter tersebut mempunyai kesan sendiri ke atas penghasilan, namun terpaksa berinteraksi antara satu sama lain disebabkan nilai-nilai rawak yang digunakan dalam eksperimen. Analisis ringkas tidak dapat dilakukan lalu Minitab digunakan dalam analisis dan mewujudkan keadaan yang optimum.

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## LIST OF SYMBOLS

| | | |
|---|---|---|
| *W/v* | - | weight over volume |
| *U* | - | Enzyme activity |
| *OD* | - | optical density |
| *µmole* | - | $1 \times 10^{-6}$ mole |
| *Rpm* | - | revelations per minute, speed |
| $R^2$ | - | standard deviation |
| *y (std.curve)* | - | optical density |
| *m* | - | Slope of graph |
| *Abs* | - | Absorbance |
| *Nm* | - | nanometers, spectrum wavelength unit |
| T | - | Temperature |
| t | - | Time, min/hours |

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# CHAPTER ONE

# INTRODUCTION

## 1.1 Introduction

Proteases (proteinases, peptidases or proteolytic enzymes) are enzymes that break peptide bonds between amino acids of proteins. The process is called *proteolytic cleavage*, a common mechanism of activation or inactivation of enzymes especially involved in blood coagulation or digestion. The field of protease research is enormous. Barrett and Rawlings (2003) estimated that approximately 8000 papers related to this field are published each year. Protease constitutes one of the most important groups of industrial enzymes accounting for about 60% of the total worldwide enzyme sales. (Olajuyigbe & Ajele, 2005; Nascimento and Martins, 2004; Beg and Gupta, 2003; Ellaiah et al., 2003).

Bacterial proteases are the most significant, compared with animal and fungal proteases and among bacteria, *Bacillus* species, as in *Bacillus subtilis* are specific producers of extracellular proteases (Olajuyigbe & Ajele, 2005).Extracellular proteases are important for the hydrolysis of proteins in cell-free environments. Therefore the production of extracellular proteases are taken in consideration for this project.

In the effort to produce extracellular proteases, the appropriate and most suitable source is determined. To produce enzymes, is source, the microorganism, be it bacteria or a fungus, will be a mesophile. As in this case bacteria are the microorganism, therefore the bacteria were mesophiles.

## 1.2 Problem Statement

Industrial applications nowadays mostly rely on catalysts to quicken production rates. Biotechnology breakthrough of the 21st century has caused many industries to move towards biocatalysts due to their favorable characteristics as a catalyst compared to the harsh chemicals often used before. The enzyme, as a renowned biocatalyst industry expands as demands increase. The prices of enzyme soar high, especially protease, so alternatives to self-produce enzyme (protease) is pursued in the midst of the situation.

## 1.3 Objective of study

The main objective of the research is to study the production of extracellular protease using *Bacillus subtilis* at pH 8.0 since it was found that maximum protease reaction occurred at this pH based on research by Olajuyigbe and Ajele (2005).

## 1.4 Scope of Study

Three scopes of study have been determined in this study that is:-

1. To study on the effect of temperature (27, 30, 37, 45 and 48°C) towards production of protease.
2. To study on the effect of agitation (129,150, 200, 250,270 rpm) towards production of protease.
3. To optimize the experimental condition using the Minitab software, through DOE (design of experiment).

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Enzyme

An **enzyme** is a protein that catalyzes, or speeds up, a chemical reaction. The word comes from the Greek word ένζυμο, *énsymo*, which comes from *én* ("at" or "in") and *simo* ("leaven" or "yeast"). Certain RNAs also have catalytic activity, but to differentiate them from protein enzymes, they are referred to as RNA enzymes or ribozymes .Enzymes are essential to sustain life because most chemical reactions in biological cells would occur too slowly, or would lead to different products, without enzymes. A malfunction (mutation, overproduction, underproduction or deletion) of a single critical enzyme can lead to a severe disease. Like all catalysts, enzymes work by lowering the activation energy of a reaction, thus allowing the reaction to proceed much faster. Enzymes may speed up reactions by a factor of many millions. An enzyme, like any catalyst, remains unaltered by the completed reaction and can therefore continue to function. Because enzymes, like all catalysts, do not affect the relative energy between the products and reagents, they do not affect equilibrium of a reaction. (http://en.wikipedia.org/wiki/Enzyme)

However, the advantage of enzymes compared to most other catalysts is their sterio-, regio- and chemo selectivity and specificity. While all enzymes have a biological role, some enzymes are used commercially for other purposes. Many household cleaners use enzymes to speed up chemical reactions (i.e., breaking down protein or starch stains in clothes).More than 5,000 enzymes are known**.** Typically the suffix *-ase* is added to the name of the substrate (*e.g.*, lactase is the enzyme that catalyzes the cleavage of lactose) or

the type of reaction (*e.g.,* DNA polymerase catalyzes the formation of DNA polymers) **(nomenclature).** However, this is not always the case, especially when enzymes modify multiple substrates. For this reason Enzyme Commission or EC numbers (International Union of Biochemistry and Molecular Biology) are used to classify enzymes based on the reactions they catalyze. Even this is not a perfect solution, as enzymes from different species or even very similar enzymes in the same species may have identical EC numbers.

By common convention, an enzyme's name consists of a description of what it does, with the word ending in *-ase*. Examples are alcohol dehydrogenase and DNA polymerase. Kinases are enzymes that transfer phosphate groups. This results in different enzymes with the same function having the same basic name; they are therefore distinguished by other characteristics, such as their optimal pH (alkaline phosphatase) or their location (membrane ATPase). Furthermore, the reversibility of chemical reactions means that the normal physiological direction of an enzyme's function may not be that observed under laboratory conditions. (http://en.wikipedia.org/wiki/Enzyme)

The International Union of Biochemistry and Molecular Biology has developed a nomenclature for enzymes, the EC numbers; each enzyme is described by a sequence of four numbers, preceded by "EC". The first number broadly classifies the enzyme based on its mechanism:

The toplevel classification is

- EC 1 *Oxidoreductases*: catalyze oxidation/reduction reactions
- EC 2 *Transferases*: transfer a functional group (e.g. a methyl or phosphate group)
- EC 3 *Hydrolases*: catalyze the hydrolysis of various bonds
- EC 4 *Lyases*: cleave various bonds by means other than hydrolysis and oxidation
- EC 5 *Isomerases*: catalyze isomerization changes within a single molecule
- EC 6 *Ligases*: join two molecules with covalent bonds.

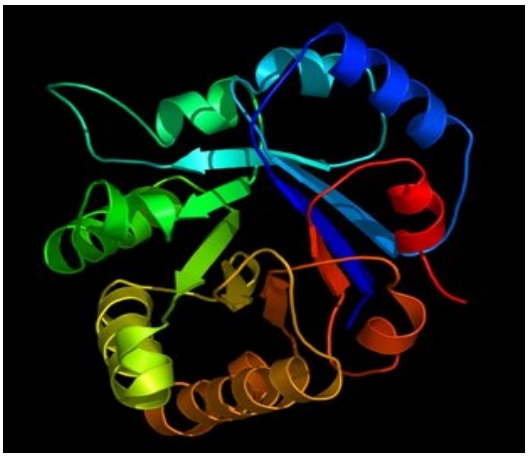

**Figure 2.1 Ribbon diagram of a catalytically perfect enzyme Triosephosphateisomerase (TIM)**

## 2.2 Production of Enzyme

With the increasing legislation demand on industry to limit the uses of harmful chemicals, the focus on enzymes for the development of environmentally friendly technologies has received considerable attention over the past few years. However, to achieve practical production levels of these proteins, the use of genetically modified microorganisms is essential. Exploitation of recombinant DNA technology and large-scale fermentation processes has enabled useful proteins to be produced in quantities that might otherwise have been difficult, if not impossible to obtain from natural resources. Consequently with the help of molecular cloning techniques a number of enzyme production systems have evolved.

One of the most well-known and used host organisms today is the Gram-negative bacterium *Escherichia coli.* Advantages when using *E. coli* are high growth rate, ability to grow to high cell densities, and the numerous expression systems and strains developed for recombinant production purposes, leading to high production rates of target proteins. Potential disadvantages include the presence of proteases, the lack of post-translational modification ability and the lack of a natural secretion pathway into the medium. These disadvantages have resulted in the development and use of a number of alternative host

systems using e.g. Gram-positive bacteria (*Bacillus* or *Lactobacillus*), yeasts (*Saccharomyces, Pichia*), insect cells, and filamentous fungi (*Aspergillus, Trichoderma*).

As a Gram-positive, *Bacillus subtilis* lacks the outer membrane, and is often an efficient protein secretor, which can be used to direct production to the medium during the process. A drawback with this organism is the often low production levels. The yeast *Saccharomyces cerevisiae* has the advantage to be a well-characterised microorganism that is classified as a GRAS (generally regarded as safe) organism. A drawback is also here the often relatively lower expression levels, and sometimes the extensive glycosylation of the target protein. When it comes to expression levels, *Pichia* is often a more feasible alternative, and a number of vectors are available.

### 2.2.1 Enzyme Unit Definition

The amount of an enzyme that will catalyze the transformation of $10^{-6}$ mole of substrate per minute or, when more than one bond of each substrate is attacked, $10^{-6}$ of 1 gram equivalent of the group concerned, under specified conditions of temperature, substrate concentration, and pH number.
The enzyme unit (U) is a unit for the amount of a particular enzyme. One U is defined as that amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute. The conditions also have to be specified: one usually takes a temperature of 30Â°C and the pH value and substrate concentration that yield the maximal substrate conversion rate.

The enzyme unit was adopted by the International Union of Biochemistry in 1964. Since the minute is not an SI unit, the enzyme unit is discouraged in favour of the katal, the unit recommended by the General Conference on Weights and Measures in 1978 and officially adopted in 1999. One katal is the amount of enzyme that converts 1 mole of substrate per second, so

1 U = 1/60 micro katal = 16.67 nano katal.

The enzyme unit should not be confused with the International Unit (IU), an unrelated measure of biologically active substances.

## 2.3 Protease Enzyme

Proteases (EC 3.4) are currently classed in six classes, serine proteases, threonine proteases, cysteine proteases, aspartic acid proteases, metalloproteases and glutamic acid proteases. These proteases, [serine protease (EC 3.4.21), cysteine(thiol) protease (EC 3.4.22), aspartic proteases (3.4.23) and metalloprotease (EC 3.4.24)] constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market (refer Chapter 1). Proteases occur naturally in all organisms and constitute 1-5% of the gene content. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g. the blood clotting cascade, the complement system and the invertebrate prophenoloxidase activating cascade). (http://en.wikipedia.org/wiki/Protease)

Peptidases can break either specific peptide bonds (*limited proteolysis*), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (*unlimited proteolysis*). The activity can be a destructive change abolishing a protein's function or digesting it to its principal components, it can be an activation of a function or it can be a signal in a signaling pathway.

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Proteolytic enzymes are very important in digestion as they breakdown the the peptide bonds in the protein foods to liberate the amino acids needed by the body. Additionally, proteolytic enzymes have been used for a long time in various forms of therapy. Their use in medicine is notable based on several clinical studies indicating their benefits in oncology, inflammatory conditions, blood rheology control, and immune regulation.

Protease is able to hydrolyze almost all proteins as long as they are not components of living cells. Normal living cells are protected against lysis by the inhibitor mechanism. Parasites, fungal forms, and bacteria are protein. Viruses are cell parasites consisting of nucleic acids covered by a protein film. Enzymes can break down undigested protein, cellular debris, and toxins in the blood, sparing the immune system this task. The immune system can then concentrate its full action on the bacterial or parasitic invasion. (www.enzymeessentials.com).

### 2.3.1 Extracellular Protease

Microorganisms elaborate a large array of proteases, which are intracellular and/or extracellular. Intracellular proteases are important for various cellular and metabolic processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Whereas, extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products. (Kaliz, 1988; Gupta, *et al*., 2002)

In addition, extracellular protease which is a hydrolytic enzyme can be classified further into two categories which are exopeptidases that cleave off amino acids from ends of protein chain, and endopeptidases that cleave peptide bonds within the protein (Rakshit and Haki, 2003). Microbial proteases are subdivided into various groups, dependent on whether they are active under acidic, neutral, or alkaline conditions and on the characteristics of the active site group of the enzyme. In production of extracellular protease, protease enzymes are most active in temperature ranges from 40-55°C.

### 2.3.2 Industrial Applications of Protease

Here are a few industrial applications favored by the protease enzyme**.** Protease enzymes are used throughout the world in these industries.

**Table 2.1 Industrial Applications of Protease Enzyme**

| **Application** | **Uses** |
|---|---|
| Biological Detergent | Used for presoak conditions and direct liquid applications helping with removal of protein stains from clothes. |
| Baking Industry | Biscuit manufacturers use them to lower the protein level of flour. |
| Brewing industry | Split polysaccharides and proteins in the malt |
| | Remove cloudiness during storage of beers. |
| Photographic industry | Dissolve gelatin off the scrap film allowing recovery of silver present |

## 2.4 Production of Protease

Proteases occur naturally in all organisms and constitute 1-5% of the gene content. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g. the blood clotting cascade, the complement system and the invertebrate prophenoloxidase activating cascade). However, as with the increase of the industrial demand, the market of protease enzyme has widened and alternate methods of producing protease is being researched.

Based on previously done researches, the protease enzyme can be produced by using or from microorganisms identified as potential in producing abundant amount of protease enzymes in need for industrial purposes. These microorganisms or microbes are

such like bacteria, fungi and other microorganism classes. Cutting down from the list, under bacteria there are many species of bacteria which are capable in producing protease enzyme, in which the well known types are under the *Bacillus* sp. (Johnvesly; Naik, 2001)

The bacteria under the *Bacillus* sp. are well known for producing extracellular proteases in use as an industrial enzyme such as *Bacillus licheniformis*, *B. amyloliquefaciens* and *B. subtilis.* The production method of protease production differs based on the capacity of the enzyme wanted or needed. For a large scale production as for industrial purposes, bioreactors should be used for more efficiency and larger yield. For a small scale production or lab scale production for testing and research purposes, it is suitable to only used Erlenmeyer flasks shaken in appropriate incubators.

## 2.5 Production of extracellular protease

As the term extracellular applies, it is known as that to produce enzymes which functions outside the cell in which it originates. Be it a fungi or bacteria, the source can be mesophiles obtained everywhere. Mesophilic bacteria and fungi are usually obtained from earth soil, given there is no temperature restraint to abide to in the experimental work.As acknowledged the *Bacillus* sp are well known for production of extracellular protease. Most bacteria from the *Bacillus* species can be obtained from earth soil therefore are preferred to be used in production of extracellular enzymes. As the title of the research implies, the aim is to produce extracellular protease therefore any mesophilic bacterium in the *Bacillus* species are favorable in terms of extracellular proteases. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments. (Nascimento; Martins, 2004)

Most certainly, in terms of protease, as well known for industrial purposes the *Bacillus* sp. is a major producer for alkaline, extracellular proteases. The challenge of producing home-made enzymes through the usage of bacteria is a challenge must be faced to no longer rely to others for enzyme supplies, be it any type of enzyme.

## 2.6 *Bacillus subtilis*

***Bacillus subtilis*** is a catalase-positive bacterium that is commonly found in soil, belonging to the genus *Bacillus*. Like other such species, it has the ability to form a tough, protective endospore, which it allows it to tolerate extreme environmental conditions. Unlike several other well-known species, *B. subtilis* is an obligate aerobe.

*B. subtilis* is not considered a human pathogen, but it can contaminate food and rarely causes food poisoning. Its spores can survive the extreme heating that is often used to cook food, and it is responsible for causing ropiness in spoiled bread. Nevertheless, a strain of *B. subtilis* formerly known as *Bacillus natto* is used in the commercial production of the Japanese delicacy natto. Other strains of *B. subtilis* have other commercial applications. For instance, *B. subtilis* strain QST 713 (marketed as QST 713 or Serenade™) has a natural fungicidal activity, and is employed as a biological control agent [1].

*B. subtilis* has proven highly amenable to genetic manipulation, and has therefore become widely adopted as a model organism for laboratory studies, especially of sporulation, which is a simplified example of cellular differentiation. In terms of popularity as a laboratory workhorse *B. subtilis* can be considered the Gram-positive equivalent of *Escherichia coli*, an extensively studied Gram-negative rod.


**Figure 2.2 a Close Look at *Bacillus Subtilis***

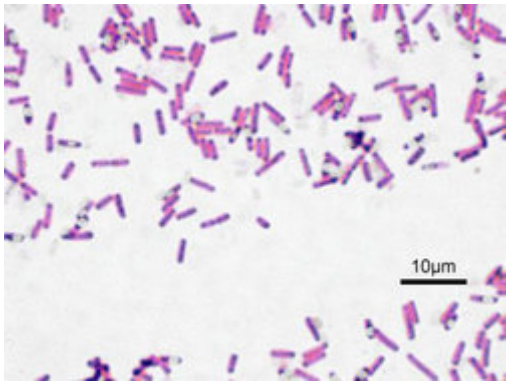



**Figure 2.3 Gram-stained *Bacillus subtilis***

| | |
|---|---|
| Kingdom: | Bacteria |
| Phylum: | Firmicutes |
| Class: | Bacilli |
| Order: | Bacillales |
| Family: | Bacillaceae |
| Genus: | *Bacillus* |
| Species: | ***B. subtilis*** |

**Figure 2.4 Scientific classifications of *Bacillus subtilis***

### 2.6.1 Facts about *Bacillus subtilis*

**Table 2.2 Facts about *Bacillus subtilis***

| | |
|---|---|
| **BioHazard Level** | 1 |
| **Growth Temperature** | 30$^{o}$C |
| **Appropriate growth media:** | nutrient agar, nutrient broth |
| **Genomic sources for** | *Bsu*15I, *Bsu*36I, *Bsu*RI, *Bsu*TUI |

| | |
|---|---|
| **restriction enzymes** | |
| **Gram Stain:** | Positive |
| **Respiration:** | Aerobic |
| **Motility:** | Yes |
| **Industrial implications:** | *B. subtilis* is one of the most widely used bacteria for the production of enzymes and specialty chemicals. Industrial applications include production of amylase, protease, inosine, ribosides, and amino acids. |
| **Misc.:** | *B. subtilis* was the first Gram-positive bacterium to be sequenced. The sequence was published in Nature in November 1997. |
| **Human health:** | Although not considered a human pathogen, B. subtilis can contaminate food and cause food poisoning in rare cases. |

**(Source:** http://www.thelabrat.com/restriction/sources/Bacillussubtilis.shtml)

## 2.7 Production of extracellular protease using *Bacillus subtilis*

Extracellular proteases are produced from microbes, favorably from bacteria of the *Bacillus sp.* In the genus of *Bacillus*, *Bacillus subtilis* is known to be a good source of producing extracellular proteases. The methodology of producing extracellular protease from *Bacillus subtilis* is the same as to any other bacteria, only in which the properties and characteristicsof the *Bacillus subtilis* must be taken in consideration for producing extracellular protease. The temperature withstanding of the bacteria also must be known, though mostly are mesophiles except those from hot or warm springs.

The proteases produced from the *Bacillus subtilis* should be make sure to function outside of the bacteria cell and not affect the bacteria itself to be said extracellular. However, most journals read produce extracellular proteases from microbes; rarely do they produce intracellular proteases, unless there were further researches related to producing intracellular protease. Should that be the matter, modifications of the methodology have been done. Otherwise stated, all proteases produced were of extracellular nature, regardless the source.

Many researches have been carried out by producing extracellular proteases from bacteria of the *Bacillus* genus, including *Bacillus subtilis.* Therefore, the production of extracellular protease from *Bacillus subtilis* is possible. Bottom-line, previous theories have been proven in respect of the matter through numerous reseaches, it is now our turn to prove it practically ourselves as a challenge in enzyme production.

## 2.8 Spectrophotometry Principles

A spectrophotometer consists of two instruments, namely a spectrometer for producing light of any selected color (wavelength), and a photometer for measuring the intensity of light. The instruments are arranged so that liquid in a cuvette can be placed between the spectrometer beam and the photometer. The amount of light passing through the tube is measured by the photometer. The photometer delivers a voltage signal to a display device, normally a galvanometer. The signal changes as the amount of light absorbed by the liquid changes.

If development of color is linked to the concentration of a substance in solution then that particular concentration can be measured by determining the extent of absorption of light at the appropriate wavelength. For example hemoglobin appears red because the hemoglobin absorbs blue and green light rays much more effectively than red. The degree of absorbance of blue or green light is proportional to the concentration of hemoglobin.

The O.D. (optical density) is directly proportional to the concentration of the colored compound. Most spectrophotometers have a scale that reads both in O.D. (absorbance) units, which is a logarithmic scale, and in % transmittance, which is an arithmetic scale. As suggested by the above relationships, the absorbance scale is the most useful for colorimetric assays.

## 2.9 Protein Absorbance Wavelength

Proteins in solution absorb ultraviolet light with absorbance maxima at 280 and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm. Secondary, tertiary, and quaternary structure all affect absorbance, therefore factors such as Ph, ionic strength, etc. can alter the absorbance spectrum.

## 2.10 Amino Acids

In chemistry, an amino acid is any molecule that contains both amine and carboxyl functional groups. In biochemistry, this shorter and more general term is frequently used to refer to alpha amino acids: those amino acids in which the amino and carboxylate functionalities are attached to the same carbon, the so-called α–carbon. These amino acids are used as the basic components of proteins. There are twenty "standard" amino acids used by cells in protein biosynthesis that are specified by the general genetic code. A list of standard amino acids describes their chemical structures and basic physical and chemical properties.

An amino acid residue is what is left of an amino acid once a molecule of water has been lost (an $H^+$ from the nitrogenous side and an $OH^-$ from the carboxylic side) in the formation of a peptide bond, the chemical bond that links the amino acid monomers in a protein chain. Each protein has its own unique amino acid sequence that is known as its primary structure. Just as the letters of the alphabet can be combined in different ways to form an endless variety of words, amino acids can be linked together in varying sequences to form a huge variety of proteins. The unique shape of each protein determines its function in the body.

Amino acids are the basic structural building units of proteins. They form short polymer chains called peptides or polypeptides which in turn form structures called proteins. The process of such formation from an mRNA template is known as translation, which is part of protein synthesis.

Twenty amino acids are encoded by the standard genetic code and are called proteinogenic or standard amino acids. The mean mass of the standard amino acids, weighted by abundance in proteins, is roughly 111 Daltons (Senko 1995). Combinations of these amino acids produce every single essential protein for the homeostasis of the human body. At least two others are also coded by DNA in a non-standard manner as follows:

- Selenocysteine is incorporated into some proteins at a UGA codon, which is normally a stop codon.
- Pyrrolysine is used by some methanogenic bacteria in enzymes that they use to produce methane. It is coded for with the codon UAG.

Other amino acids contained in proteins are usually formed by post-translational modification, which is modification after translation in protein synthesis. These modifications are often essential for the function of the protein. Some of the 20 standard proteinogenic amino acids are called essential amino acids because the human body cannot synthesize them from other compounds through chemical reactions, and they therefore must be obtained from food. Histidine and arginine are generally only considered essential in children, because the metabolic pathways that synthesize these amino acids are not fully developed in children.

| **Essential** | **Nonessential** |
|---|---|
| Isoleucine | Alanine |
| Leucine | Asparagine |
| Lysine | Aspartate |
| Methionine | Cysteine |
| Phenylalanine | Glutamate |
| Threonine | Glutamine |
| Tryptophan | Glycine |
| Valine | Proline |
| Arginine* | Serine |
| Histidine* | Tyrosine |

(*) Essential only in certain cases

**Figure 2.5 Essential and non-essential Amino Acids**

## 2.11 Tyrosine

**Tyrosine** (from the Greek *tyros*, meaning *cheese*, as it was first discovered in cheese), **4-hydroxyphenylalanine**, or **2-amino-3(4-hydroxyphenyl)-propanoic acid**, is one of the 20 amino acids that are used by cells to synthesize proteins. It has a phenol side chain with a hydroxyl group. Upon the location of the hydroxyl group, there are three structural isomers of Tyr, namely para-Tyr (p-Tyr), meta-Tyr (m-Tyr) and ortho-Tyr (o-Tyr). Enzymatically, only the first isomer (p-Tyr) is produced from L-Phe by the Phe-hydroxylase enzyme. The other two isoforms, m-Tyr and o-Tyr can be produced as a consequence of free radical attack on Phe in states with increased oxidative stress.

It plays a key role in signal transduction, since it can be *tagged* with a phosphate group (phosphorylated) by protein kinases to alter the functionality and activity of certain enzymes. (In its phosphorylated state, it is sometimes referred to as **phosphotyrosine**.) Tyrosine is also precursor to the thyroid hormones *thyroxine* and *triiodothyronine*, the pigment *melanin*, and the biologically-active catecholamines *dopamine*, *norepinephrine* and *epinephrine*. In Papaver somniferum, the opium poppy, it is used to produce morphine.

Tyrosine cannot be completely synthesized by animals, although it can be made by hydroxylation of phenylalanine if the latter is in abundant supply. It is produced by plants and most microorganisms from prephenate, an intermediate on the shikimate pathway. Prephenate is oxidatively decarboxylated with retention of the hydroxyl group to give *p*-hydroxyphenylpyruvate. This is transaminated using glutamate as the nitrogen source to give tyrosine and α-ketoglutarate.

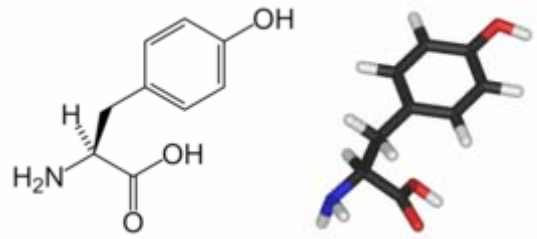
| Tyrosine | |
|---|---|
|  | |
| Systematic name | (*S*)-2-Amino-3-(4-hydroxy-phenyl)-propanoic acid |
| Abbreviations | **Tyr**<br>**Y** |
| Chemical formula | $C_9H_{11}NO_3$ |
| Molecular mass | 181.19 g $mol^{-1}$ |
| Melting point | 343 °C |
| Density | 1.456 g $cm^{-3}$ |
| Isoelectric point | 5.66 |
| p$K_a$ | 2.24<br>9.04<br>10.10 |
| CAS number | [60-18-4] |
| EINECS number | 200-460-4 |

**Figure 2.6 Properties of Tyrosine**

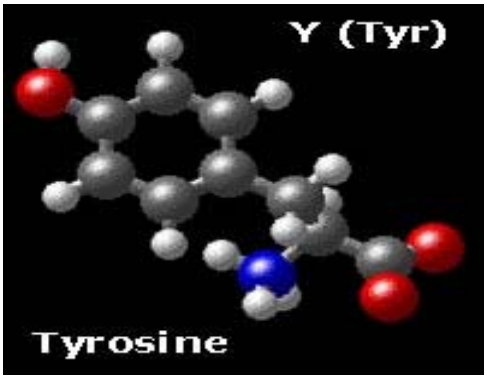



**Figure 2.7 Tyrosine Model Structure**

L-Tyrosine is sometimes recommended by practitioners as helpful for weight loss, clinical depression, and phenylketonuria; however, a study found that it had no impact on performance.L-tyrosine is a protein amino acid. It is classified as a conditionally essential amino acid.

Under most circumstances, the body can synthesize sufficient L-tyrosine, principally from L-phenylalanine, to meet its physiological demands. However, there are conditions where the body requires a dietary source of the amino acid for its physiological demands. For example, L-tyrosine is an essential amino acid for those with phenylketonuria. L-tyrosine is found in proteins of all life forms. Dietary sources of L-tyrosine are principally derived from animal and vegetable proteins. Vegetables and juices contain small amounts of the free amino acid. The free amino acid is also found in fermented foods such as yogurt and miso.

L-tyrosine is also known as beta- (para-hydroxyphenyl) alanine, alpha-amino-para-hydroxyhydrocinnamic acid and (S) – alpha-amino-4-hydroxybenzenepropanoic acid. It is abbreviated as either Tyr of by its one-letter abbreviation Y. The molecular formula of L-tyrosine is $C_9H_{10}NO_3$, and its molecular weight is 181.19 daltons. L-tyrosine is an aromatic amino acid with the following structural formula:

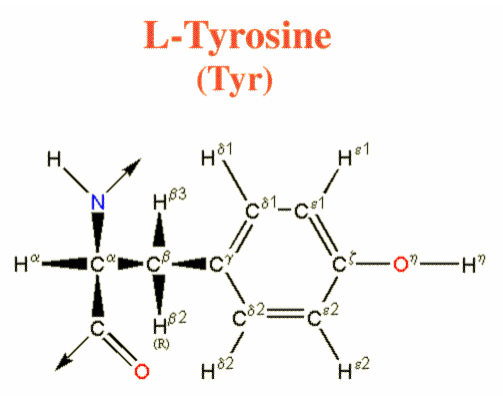



**Figure 2.8 L-Tyrosine Structure**

### 2.11.1 Tyrosine Absorbance

Tyrosine, an essential amino acid, is also an aromatic amino acid and is derived from phenylalanine by hydroxylation in the para position. While tyrosine is hydrophobic, it is significantly more soluble that is phenylalanine. The phenolic hydroxyl of tyrosine is significantly more acidic than are the aliphatic hydroxyls of either serine or threonine, having a pKa of about 9.8 in polypeptides. As with all ionizable groups, the precise pKa will depend to a major degree upon the environment within the protein. Tyrosines that are on the surface of a protein will generally have a lower pKa than those that are buried within a protein; ionization yielding the phenolate anion would be exceedingly unstable in the hydrophobic interior of a protein.

Tyrosine absorbs ultraviolet radiation and contributes to the absorbance spectra of proteins. The absorbance spectrum of tyrosine will be shown later; the extinction of tyrosine is only about 1/5 that of tryptophan at 280 nm, which is the primary contributor to the UV absorbance of proteins depending upon the number of residues of each in the protein.

### 2.11.2 Tyrosine Standard Calibration Curve

As a reference to protease enzyme activity, tyrosine standard curves are usually generated using an appropriate amount of tyrosine diluted in suitable fractions with solvents, favorably water. The suitably diluted samples will be read at a spectrophotometer at a wavelength measurement of 280 nm.

## 2.12 Design of Experiments

The first statistician to consider a methodology for the design of experiments was Sir Ronald A. Fisher. He described how to test the hypothesis that a certain lady could distinguish by flavor alone whether the milk or the tea was first placed in the cup. While

this sounds like a frivolous application, it allowed him to illustrate the most important means of experimental design:

- Randomization – The process of making something random
- Replication – repeating the creation of a phenomenon, so that the variability associated with the phenomenon can be estimated
- Blocking – the arranging of experimental units in groups (blocks) which are similar to one another
- Orthogonality – Means perpendicular, at right angles or statistically normal.
- use of factorial experiments instead of the one-factor-at-a-time method

Analysis of the design of experiments was built on the foundation of the analysis of variance, a collection of models in which the observed variance is partitioned into components due to different factors which are estimated and/or tested.

Some efficient designs for estimating several main effects simultaneously were found by Raj Chandra Bose and K. Kishen in 1940 at the Indian Statistical Institute, but remained little known until the Plackett-Burman designs were published in Biometrika in 1946.

In 1950, Gertrude Mary Cox and William Cochran published the book Experimental Design which became the major reference work on the design of experiments for statisticians for years afterwards.

## 2.13 Minitab

Minitab is a computer program designed to perform basic and advanced statistical functions. It combines the user-friendliness of Microsoft Excel with the ability to perform complex statistical analysis. In 1972, instructors at the Pennsylvania State University developed Minitab as a light version of OMNITAB, a statistical analysis program by NIST.

It soon became the world's leading statistical software for academic instruction, and is currently used at more than 4,000 colleges and universities worldwide.

The software's success led its creators to form Minitab Inc. in 1983. The privately owned company is headquartered in State College, Pennsylvania, with subsidiaries in Coventry, England (Minitab Ltd.) and Paris, France (Minitab SARL). The company also maintains a worldwide network of resellers and representatives.

Today, Minitab is often used in conjunction with the implementation of Six Sigma and other statistics-based process improvement methods. Thousands of companies in more than 80 countries use it, including over half the companies in the Fortune 500.

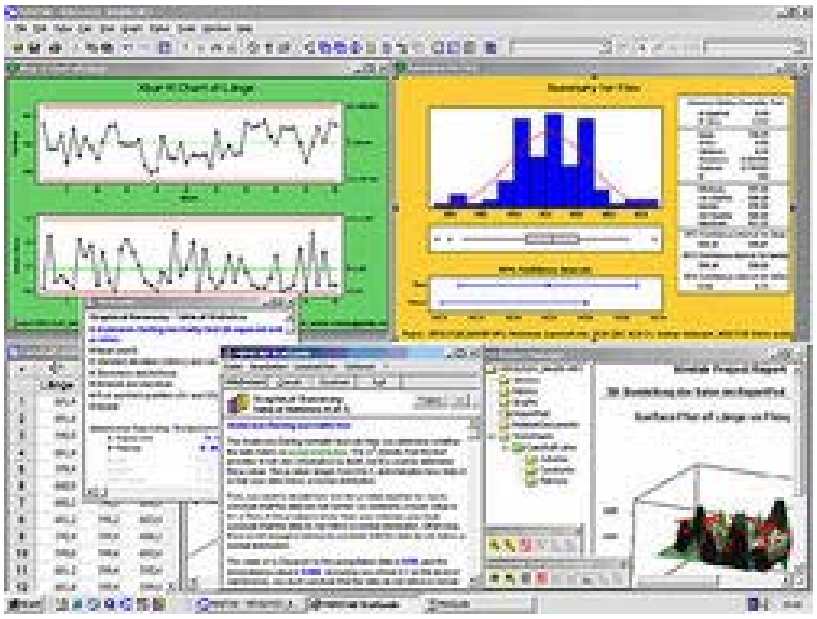


**Figure 2.9 Minitab's interface**

## 2.14 Response Surface Design

Sometimes simple linear and interaction models are not adequate. For example, suppose that the outputs are defects or yield, and the goal is to minimize defects and maximize yield. If these optimal points are in the interior of the region in which the

experiment is to be conducted, you need a mathematical model that can represent curvature so that it has a local optimum. The simplest such model has the quadratic form

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2$$

containing linear terms for all factors, squared terms for all factors, and products of all pairs of factors.

Designs for fitting these types of models are known as response surface designs. One such design is the full factorial design having three values for each input. Although the Statistics Toolbox is capable of generating this design, it is not really a satisfactory design in most cases because it has many more runs than are necessary to fit the model.The two most common designs generally used in response surface modeling are central composite designs and Box-Behnken designs. In these designs the inputs take on three or five distinct values (levels), but not all combinations of these values appear in the design.

The functions described here produce specific response surface designs:

- Central Composite Designs
- Box-Behnken Designs

## 2.15 Central Composite Designs

Central composite designs are response surface designs that can fit a full quadratic model. To picture a central composite design, imagine you have several factors that can vary between low and high values. For convenience, suppose each factor varies from -1 to +1.

One central composite design consists of cube points at the corners of a unit cube that is the product of the intervals [-1, 1], star points along the axes at or outside the cube,

and center points at the origin. Central composite designs are of three types. Circumscribed (CCC) designs are as described above. Inscribed (CCI) designs are as described above, but scaled so the star points take the values -1 and +1, and the cube points lie in the interior of the cube. Faced (CCF) designs have the star points on the faces of the cube. Faced designs have three levels per factor, in contrast with the other types, which have five levels per factor. The following figure shows these three types of designs for three factors.

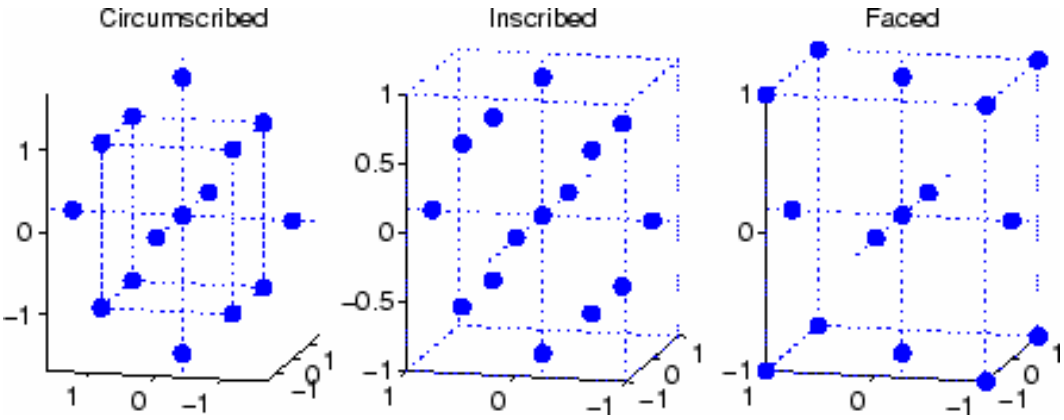



**Figure 2.10 Three types of Central Composite Design**